

Reaction of 3-dehydroecdysone with certain n.m.r. solvents

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Synthetically prepared 3-dehydroecdysone shows by n.m.r. spectroscopy a mixture of two and three components in $^2\text{H}_2\text{O}$ and $[\text{H}_4]$ methanol respectively; only 3-dehydroecdysone is indicated in $[\text{H}_5]$ pyridine. Although 3-dehydroecdysone is the sole component in $[\text{H}_5]$ pyridine, it represents only 62% and 55% in $^2\text{H}_2\text{O}$ and $[\text{H}_4]$ methanol respectively. Evidence indicates that the other component in $^2\text{H}_2\text{O}$ is a 3- $[\text{H}_2]$ hydrate of 3-dehydroecdysone, and that in $[\text{H}_4]$ methanol the other two components are isomeric $[\text{H}_3]$ hemiacetals of 3-dehydroecdysone.

INTRODUCTION

In 1974, when the first 3α -ecdysteroids, 3-epi-20-hydroxyecdysone and 3-epiecdysone, were isolated and identified from meconium fluid of the tobacco hornworm, *Manduca sexta* [1], 3-epiecdysone was also identified as the product *in vitro* of an enzyme system from *M. sexta* midgut [2]. It was then postulated that 3-dehydroecdysteroids, such as 3-dehydroecdysone, were intermediates in the conversion of the more biologically active 3β -ecdysteroids into less active 3α -ecdysteroids, and that this process afforded a means of regulating the moulting-hormone titres of insects. Although 3-dehydroecdysteroids have been synthesized enzymically and chemically some years ago [3–5], interestingly it is only recently that proof of this two-step enzymic reaction has been obtained [6–8]. Because of the important role that epimerization plays in regulating moulting-hormone titre in insects in general, this reaction is being investigated in a number of laboratories, including ours.

Recently it was reported that prothoracic glands of *M. sexta in vitro* released a mixture of 2-dehydroecdysone and 3-dehydroecdysone (1:2) [9]. This ratio, which was determined principally by n.m.r. spectroscopy performed in $^2\text{H}_2\text{O}$, was also observed for synthetically prepared 3-dehydroecdysone. This prompted us to re-examine synthetically prepared 3-dehydroecdysone. We report here that the synthetically prepared 3-dehydroecdysone does not contain 2-dehydroecdysone, although it does show a mixture of two and three compounds by n.m.r. spectroscopy in $^2\text{H}_2\text{O}$ and $[\text{H}_4]$ methanol respectively. In $[\text{H}_5]$ pyridine, however, only 3-dehydroecdysone is indicated.

MATERIALS AND METHODS

Preparation of 3-dehydroecdysone

Ecdysone (75 mg) in 26 ml of diglyme and 11 ml of water was oxidized as previously reported [4,5]. The material after work-up was chromatographed over 8 g of chloroform/benzene-(17:3, v/v)-washed silica gel. The material was placed on the column in this solvent and the

following fractions were collected: 100 ml fraction of chloroform/benzene (17:3, v/v), and 50 ml fractions of successively 5% (v/v) benzene in chloroform, chloroform and 5%, 10%, 15%, 15% and 20% (v/v) ethanol in chloroform. The chromatography was monitored by t.l.c., and the fraction eluted with 10% ethanol in chloroform when recrystallized from methanol/ethyl acetate gave 34 mg of 3-dehydroecdysone, m.p. 196–199 °C (decomp.). The mother liquor contained a small quantity of 3,22-didehydroecdysone. The first fraction eluted with 15% ethanol in chloroform gave 10 mg of a mixture of ecdysone and a small amount of 3-dehydroecdysone. The second fraction eluted with 15% ethanol in chloroform gave 25 mg of ecdysone.

N.m.r. spectroscopy

^1H -n.m.r. spectra were obtained at 400.13 MHz and 293 K on a Bruker WH400 spectrometer, typically with 128 transients for a 0.5–1.0 mg sample. A Lorentzian-to-Gaussian window function was used. Referencing was to tetramethylsilane with the solvent residual proton resonance as a secondary standard (4.75 p.p.m. for $^2\text{H}_2\text{O}$). Peak integrals were obtained after careful spline baseline correction. Two-dimensional COSY-45 spectra [10] were obtained overnight, with 2048 data points and 512 time increments. They were transformed with sine-bell windows in both dimensions and then symmetrized.

The D-ring resonances in $[\text{H}_5]$ pyridine were assigned via n.O.e. difference spectroscopy, with irradiation at the C-18 methyl protons [11].

RESULTS

One-dimensional ^1H and two-dimensional COSY-45 n.m.r. spectra of 3-dehydroecdysone (I) were first obtained in $[\text{H}_4]$ methanol. It was immediately apparent from the less crowded regions of the one-dimensional spectrum (Fig. 1), notably the H-7 region at 5.8–6.0 p.p.m. and the methyl region at 0.7–1.3 p.p.m., that three similar compounds, here labelled (I), (Ib) and (Ic), were present in this solvent, in the proportions 55:27:18.

Abbreviations used: COSY, correlation spectroscopy; n.O.e., nuclear Overhauser enhancement.

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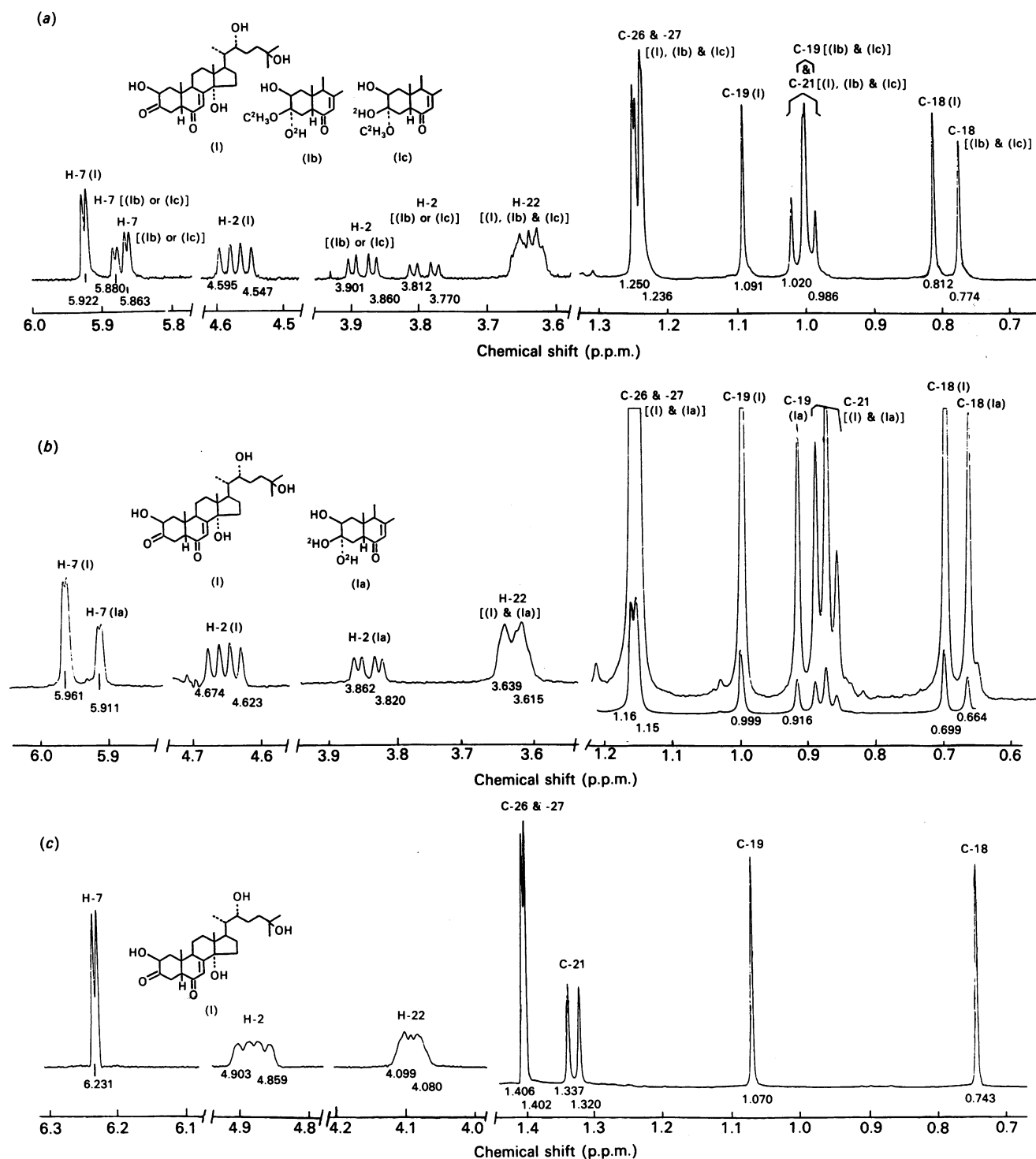


Fig. 1. Partial ^1H -N.M.R. spectra of 3-dehydroecdysone (I) taken in (a) $[\text{D}_4]\text{methanol}$, (b) D_2O and (c) $[\text{D}_5]\text{pyridine}$

C = methyl. Structures of compounds (Ib) and (Ic) may be reversed.

With this information, it proved possible to analyse other resonances, and in particular to note that H-2 in the major component appears at 4.57 p.p.m., whereas the same proton in the two minor components, in descending order of abundance, resonates at 3.88 and 3.79 p.p.m. The 4.57 p.p.m. shift is typical of $\text{CH}(\text{OX})$ proton flanked by a keto group, whereas 3.8 p.p.m. suggests $\text{CH}(\text{OX})$ flanked by a saturated carbon atom. However, the

couplings to H-2 are: compound (I), $J = 12.5$ Hz, d, and $J = 6.4$ Hz, d; compound (Ib), $J = 12.0$ Hz, d, and $J = 4.6$ Hz, d; compound (Ic), $J = 12.2$ Hz, d, and $J = 4.5$ Hz, d. This showed that the difference between the three compounds is probably not due to any change in the number or orientation of the C-H bonds vicinal to H-2.

The two-dimensional COSY-45 spectrum of 3-dehydroecdysone obtained in $[\text{D}_4]\text{methanol}$, a portion

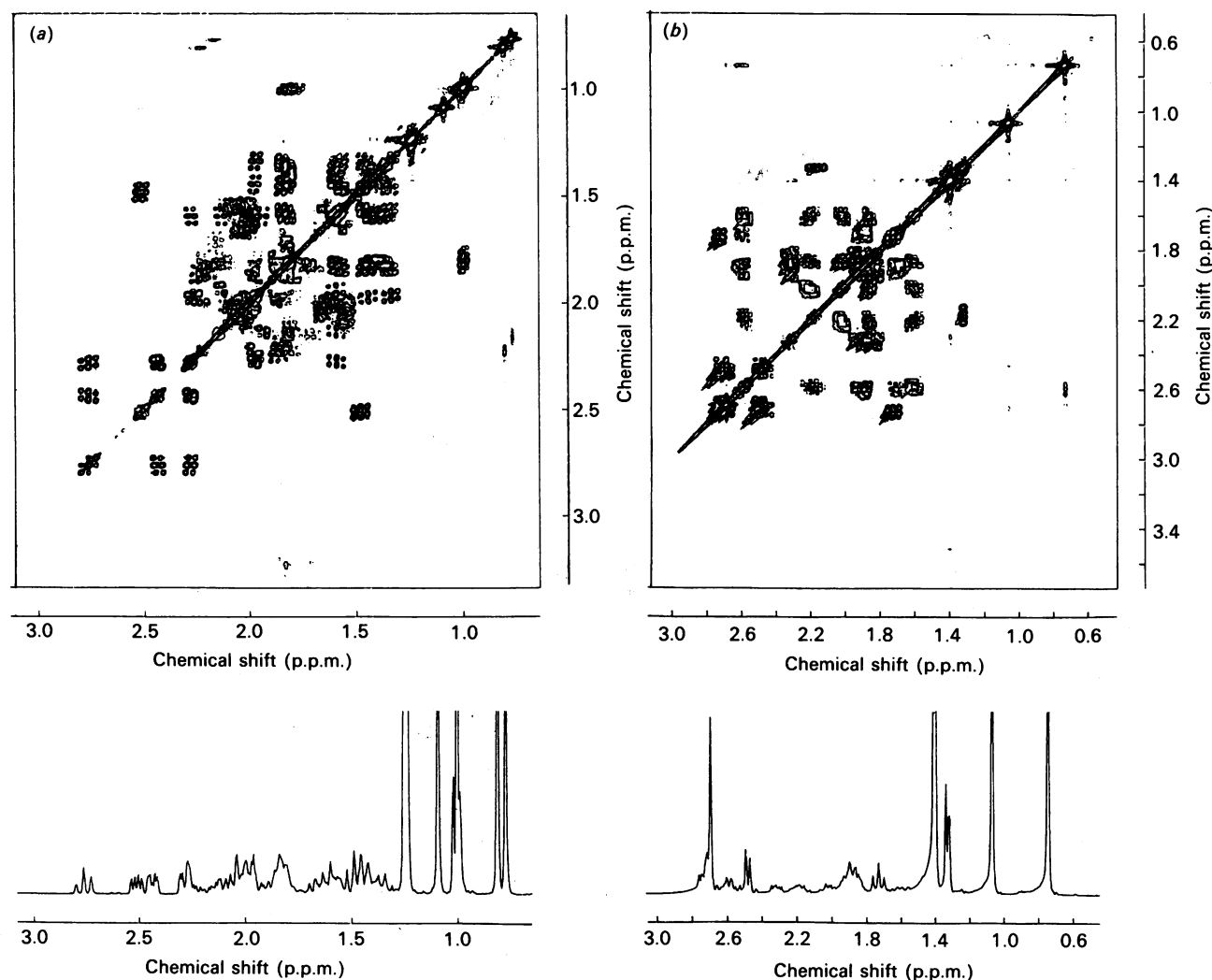


Fig. 2. Partial one-dimensional and two-dimensional COSY-45 n.m.r. spectra of 3-dehydroecdysone (I) in (a) $[\text{}^2\text{H}_4]\text{methanol}$ and (b) $[\text{}^2\text{H}_5]\text{pyridine}$

of which is shown in Fig. 2(a), revealed a similar pattern for other resonances, all of which could be assigned with sufficient confidence to rule out tautomers. In particular, only the resonances from protons in or near ring A show significant chemical-shift separation between compounds (I), (Ib) and (Ic) for a given proton. Furthermore, the separations between compounds (Ib) and (Ic) are consistently smaller than those between compound (I) and compound (Ib) or compound (Ic). These assignments are given in Table 1.

Many of the coupling constants in the three compounds may also be approximately gauged from the two-dimensional spectrum, and once again it is clear from their similarity that no major conformational change distinguishes the three compounds.

A logical explanation of the above observation is that compound (I) is the 3-dehydroecdysone as formulated, whereas compounds (Ib) and (Ic) are isomeric $[\text{}^2\text{H}_3]\text{hemiacetals}$. Compound (Ib) is the more abundant of these. However, the formulae given for compounds (Ib) and (Ic) in Fig. 1 should be regarded as interchangeable, because our data cannot complete this assignment.

The results immediately suggested two further

experiments as a test of the above explanation. Firstly, the corresponding spectrum of compound (I) obtained in $^2\text{H}_2\text{O}$ should show only two species, namely the 3-dehydro form and the 3- $[\text{}^2\text{H}_2]\text{hydrate}$. Similarly, the spectrum in an aprotic solvent such as pyridine should only show the 3-dehydro form. The spectral data for 3-dehydroecdysone in $^2\text{H}_2\text{O}$ and $[\text{}^2\text{H}_5]\text{pyridine}$ confirm this precisely (Fig. 1), and are supported by two-dimensional COSY-45 spectra (see Fig. 2b). All protons for compound (I) in $[\text{}^2\text{H}_5]\text{pyridine}$ could be assigned, although n.O.e. difference spectroscopy was required for assignment of protons in the D ring. The proton assignments are presented in Table 1. Only the D-ring resonances (H-15–17) remain unassigned in the methanol and water solutions. The proportion of compound (I) in $^2\text{H}_2\text{O}$ at 293 K is 62%, indicating that the 3-dehydro structure is the major form in all cases, even when $[\text{}^2\text{H}_4]\text{methanol}$ or $^2\text{H}_2\text{O}$ is present in such high molarity.

DISCUSSION

Our results indicate clearly that the methods of synthesis and purification of 3-dehydroecdysone by common chromatographic procedures yield spectroscopically pure

Table 1. ^1H -n.m.r. data for 3-dehydroecdysone (I) and/or its solvent adduct(s)

| ^1H | Chemical shift (p.p.m.) | | | | | |
|--------------------|--------------------------|------------------------|------|-------------------------|-------|-------|
| | $[\text{H}_5]$ -Pyridine | $^2\text{H}_2\text{O}$ | | $[\text{H}_4]$ Methanol | | |
| | (I) | (I) | (Ia) | (I) | (Ib)* | (Ic)* |
| 1a | 1.72 | 1.49 | 1.24 | 1.48 | 1.33 | 1.44 |
| 1b | 2.73 | 2.45 | 1.94 | 2.51 | 1.97 | 1.98 |
| 2 | 4.88 | 4.65 | 3.84 | 4.57 | 3.88 | 3.79 |
| 4a | 2.48 | 2.27 | 1.72 | 2.28 | 1.98 | 1.93 |
| 4b | 2.70 | 2.64 | 1.66 | 2.76 | 1.59 | 1.59 |
| 5 | 2.70 | 2.49 | 2.26 | 2.43 | 2.28 | 2.14 |
| 7 | 6.23 | 5.96 | 5.91 | 5.92 | 5.87 | 5.88 |
| 9 | 3.89 | 3.38 | 3.05 | 3.54 | 3.21 | 3.26 |
| 11a | 1.69 | 1.75 | 1.64 | 1.82 | 1.72 | 1.72 |
| 11b | 1.91 | 1.90 | 1.80 | 1.97 | 1.84 | 1.84 |
| 12a | 1.87 | 1.59 | 1.55 | 1.87 | 1.82 | 1.82 |
| 12b | 2.60 | 2.00 | 1.95 | 2.21 | 2.13 | 2.13 |
| 15a | 2.02 | † | † | † | † | † |
| 15b | 1.86 | † | † | † | † | † |
| 16a | 2.19 | † | † | † | † | † |
| 16b | 1.60 | † | † | † | † | † |
| 17 | 2.58 | † | † | † | † | † |
| 20 | 2.16 | 1.73 | 1.73 | 1.80 | 1.80 | 1.80 |
| 22 | 4.09 | 3.63 | 3.63 | 3.62 | 3.62 | 3.62 |
| 23a | 1.90 (23) | 1.45 | 1.45 | 1.58 | 1.58 | 1.58 |
| 23b | 1.90 (23') | 1.29 | 1.29 | 1.36 | 1.36 | 1.36 |
| 24a | 2.31 (24) | 1.37 | 1.37 | 1.45 | 1.45 | 1.45 |
| 24b | 1.80 (24') | 1.65 | 1.65 | 1.82 | 1.82 | 1.82 |
| 18-CH ₃ | 0.743 | 0.70 | 0.66 | 0.81 | 0.77 | 0.77 |
| 19-CH ₃ | 1.070 | 1.00 | 0.92 | 1.09 | 1.01 | 1.01 |
| 21-CH ₃ | 1.329 | 0.88 | 0.87 | 1.01 | 0.99 | 0.99 |
| 26-CH ₃ | 1.402 | 1.15 | 1.15 | 1.24 | 1.24 | 1.24 |
| or | | | | | | |
| 27-CH ₃ | 1.406 | 1.16 | 1.16 | 1.25 | 1.25 | 1.25 |

* These assignments may be reversed.
† Not assigned here.

3-dehydroecdysone (I) as evidenced by one-dimensional and two-dimensional n.m.r. spectral data obtained in $[\text{H}_5]$ pyridine (Figs. 1 and 2b). It should be mentioned that n.m.r. spectra obtained in $[\text{H}_5]$ pyridine of the first chemically synthesized 3-dehydroecdysone [4,5] also showed methyl resonance values similar to those reported

here. Our results further indicate, however, that 3-dehydroecdysone reacts with commonly used n.m.r. solvents such as $[\text{H}_4]$ methanol and $^2\text{H}_2\text{O}$ and shows a mixture of compounds. The proportion of unmodified 3-dehydroecdysone (I) (62%) in $^2\text{H}_2\text{O}$ agrees closely with the 66% previously observed in this solvent for synthetic 3-dehydroecdysone [9]. The n.m.r. spectrum of 3-dehydroecdysone obtained in $^2\text{H}_2\text{O}$ also resembles the published spectrum [9] (see Fig. 5, particularly inserts A and B, of that paper). Thus it is likely that the compound believed to be 2-dehydroecdysone is in fact the 3- $[\text{H}_2]$ hydrate (Ia) reported here, and that their synthetic material is perhaps wholly compound (I). A similar conclusion may be applicable to the presumed mixture of compounds released *in vitro* by the prothoracic glands of *M. sexta* [9].

The reaction of 3-dehydroecdysone with $^2\text{H}_2\text{O}$ gives 38% of the product (Ia). In $[\text{H}_4]$ methanol the total percentage of compounds (Ib) and (Ic) is 45%. All three products are unstable and appear to exist only in solution. They are reconverted into compound (I) simply by removing the solvent under vacuum or by a stream of N_2 . The proportions of hemiacetal and (particularly) hydrate formed are substantially higher than with many simple ketones, probably because of hydrogen-bonding to the 2β -hydroxy group. It is thus recommended that, for compounds of unknown structure, spectral data should be obtained in at least two different solvents.

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